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Stable Ozonides with Vitamin E Acetate versus Corticosteroid in the Treatment of Lichen Sclerosus in Foreskin: Evaluation of Effects on Inflammation

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Keywords
Lichen sclerosus · Phimosis · Children · Stable ozonides · Vitamin E acetate · Corticosteroids

Abstract
Background: Lichen sclerosus (LS) is a disease of the skin of unclear etiology that can occur in the foreskin. Topical therapy with corticosteroids is recommended, but they can have side effects. ***Objectives:*** We aimed to compare the effects of ozonides with vitamin E acetate (OZOILE) versus topical corticosteroid in children undergoing circumcision. ***Method:*** Twenty children undergoing circumcision were treated before surgery: 10 children with OZOILE cream and 10 with 0.1% mometasone furoate once a day for 7 days. Ten age-matched patients with LS of the foreskin without any treatment were recruited as controls. Transcript levels of proinflammatory and anti-inflammatory cytokines and e-cadherin were evaluated in removed foreskins by qRT-PCR. ***Results:*** OZOILE and steroid topical treatment produced a similar reduction of TNF-α and IL-1β mRNA levels in foreskins from patients with LS when compared to untreated patients ($p < 0.001$). OZOILE and steroid treatment caused an increase in the transcript levels of IL-13 and e-cadherin in the foreskin of patients affected by LS in comparison to untreated foreskin ($p < 0.001$). ***Conclusions:*** On the basis of our biochemical data, a randomized clinical trial might be useful to verify the actual clinical effect of OZOILE as alternative treatment to corticosteroids in children affected by LS of the foreskin.

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Introduction

Lichen sclerosus et atrophicus (LS) is a chronic, inflammatory disease of the skin that preferentially localizes to anogenital region. LS was first described by Hallopeau [1]. The disease was defined with various names including LS et atrophicus and kraurosis of the vulva. In 1976, the International Society for the Study of Vulvovaginal Disease stated to call it LS [2]. LS has been described in adults and children of both sexes and it affects most frequently women than men; moreover, adults are more affected than children [3, 4]. The true incidence of LS is difficult to estimate because it is often asymptomatic.

LS in pediatric age is often misdiagnosed and can cause damaging effects. The localization of LS to foreskin has been also called balanitis xerotica obliterans. It was first described in 1928 by Stuhmer and it is characterized by white atrophic plaques that may affect the foreskin, glans penis, frenulum and meatus, or urethra, but the perianal area is often spared [5–7].

Celis et al. [8] in their review estimated the incidence of LS of foreskin to be 35%. It is considered responsible for most cases (80–90%) of acquired phimosis [9, 10].

The etiology of LS of the foreskin is still unclear and probably multifactorial. They are believed to be autoimmune diseases caused through both humoral and cell-mediated autoimmunity. In the literature, an association between LS and atopy, thyroiditis, alopecia areata, diabetes mellitus, vitiligo, pernicious anemia and celiac and Crohn's disease has been demonstrated. Moreover, it has been hypothesized that genetic predisposition leads to the development of an immune response [10–12].

LS of the penis can be complicated involving urethral meatus or urethra. In literature, it has been reported that meatal stenosis can progress to urethral strictures and renal failure due to obstructive uropathy [6, 13, 14].

The topical medical therapy, before or after surgical treatment with several drugs, has been described [15]. A recent evidence-based guideline on the treatment of anogenital LS in adults, girls, and boys states that the gold standard for treatment is the use of the very potent topical steroids [9, 16]. Moreover, corticosteroids therapy can have several effects, such as skin atrophy, risk of superinfection, xerosis, hypopigmentation, burning, irritation, and rarely, suppression of the hypothalamic-pituitary axis [17].

Topical therapy with vitamin E has been proposed as treatment maintenance of vulvar LS after corticosteroids [18]. Avocado and soybean extracts have been employed as anti-inflammatory, anti-fibrotic, emollient, and lenitive actions and are considered effective alternatives in the treatment of symptoms and signs of mild-to-moderate vulvar LS [19].

In our previous study, we evaluated the effects of the topical preoperative use of stable ozonides with vitamin E acetate (OZOILE) cream on the inflammatory status and tissue remodeling in male children with LS of the foreskin undergoing circumcision [20].

In this observational study, we compared the effects of OZOILE versus topical corticosteroid preoperative treatment in children with phimosis affected by LS undergoing circumcision, evaluating anti-inflammatory and tissue regeneration effects.

Materials and Methods

Patients Recruitment

Thirty children with phimosis undergoing circumcision and with histological diagnosis of LS were included in this observational study. The mean age at diagnosis was 9.4 ± 3.6 years, ranging from 5 to 15 years. All the patients showed no alteration in the blood count or C reactive protein at the time of surgery.

The histological diagnosis of LS was defined by pathologists as an epithelial-stromal lesion characterized by squamous atrophy or hyperplasia, band like infiltration, hyalinization of the papillar dermis, hyperkeratosis, pigment incontinence, and/or dermal edema.

The patients were divided into 3 groups of 10 each. The control group (n = 10) did not receive any medication before surgery, while the patients of the other 2 groups were treated by a pediatrician before surgery with OZOILE cream (n = 10) or 0.1% mometasone furoate (n = 10) once a day for 7 days.

Foreskin samples obtained during circumcision procedure were divided into 2 parts: one portion was submitted for histological analysis and the other for the analysis of transcript levels of pro- and anti-inflammatory cytokines and E-cadherin by qRT-PCR.

The study protocol was approved by the Ethical Committee of our hospital and all procedures performed in this study were in accordance with the ethical standards and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent and authorization to use sensitive data were obtained from the parents of all subjects at the time of admission.

Gene Expression Analysis

After sampling, foreskin tissues were immersed in 500 μL of RNA stabilization reagent (RNAlater; Life Technologies, Milan, Italy), and stored at –80 °C until RNA was isolated. Total RNA was isolated using TRIzol reagent (Life Technologies), and 2 μg of total RNA were reverse-transcribed with high-capacity cDNA Archive kit (LifeTechnologies). Then, mRNA levels of TNF-α, IL-1β, IL-13, and E-cadherin were analyzed by Sybr Green Real-Time PCR. Quantitative PCR reactions were set up in duplicate in a 96-well plate and were carried out in 10 μL reaction volume containing 1 × Sybr Green Master Mix (Life Technologies), 0.1 μM specific primers and 25 ng RNA converted into cDNA. Real-time PCR was performed in a 7900HT fast Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) with the following profile: one cycle at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and

Table 1. qRT-PCR primer sequences

Gene	Forward primer (5′→3′)	Reverse primer (5′→3′)
ACT-β	TGGTTACAGGAAGTCCCTTGCC	ATGCTATCACCTCCCCTGTGTG
TNF-α	GTGAGGAGGACGAACATC	GAGCCAGAAGAGGTTGAG
IL-1β	GCTTATTACAGTGGCAATGA	TAGTGGTGGTCGGAGATT
IL-13	GTCTCAGCTGGGCAGTTTTC	TCTGCAACTTCAATAGTCAGGTCC
E-cadherin	TGAGTGTCCCCCGGTATCTTC	CAGTATCAGCCGCTTTCAGATTTT

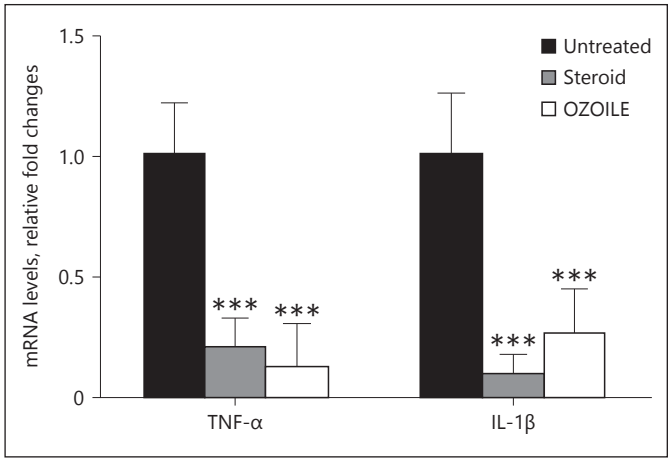

Fig. 1. Comparison of transcript levels of proinflammatory cytokines in foreskin tissues from patients affected by LS of the foreskin treated with OZOILE ($n = 10$), 0.1% mometasone furoate ($n = 10$) or untreated ($n = 10$). The results are expressed as relative fold changes versus untreated patients. The data are the means ± SEM. *** $p < 0.001$ significant differences in comparison with untreated patients. OZOILE, ozonides with vitamin E acetate.

60 °C for 1 min. A standard dissociation stage was added to assess primer specificity.

Data were analyzed using the $2^{-\Delta\Delta Ct}$ relative quantification method, and values are presented as fold change relative to the untreated group. β-actin was used as an endogenous control. The primer sequences are reported in Table 1.

Statistical Analysis

All values are expressed as mean ± standard error of the mean (SEM). Statistical analysis of gene expression data was carried out using one-way analysis of variance, followed by the post hoc Bonferroni test. p values less than 0.05 were considered significant.

Results

The expression levels of pro- and anti-inflammatory cytokines in foreskin samples obtained from LS affected patients untreated or treated with the topical creams, 0.1% mometasone furoate or OZOILE, were examined by Real-Time PCR. The treatment with both creams was able to significantly reduce the mRNA levels of the pro-inflammatory cytokines in comparison with foreskin tissues from LS-affected patients without any medications. The TNF-α and IL-1β mRNA levels were reduced by more than 70% in tissues treated with both 0.1% mometasone furoate and OZOILE compared to untreated ones ($p < 0.001$; Fig. 1). On the other hand, the expression of the anti-inflammatory cytokine IL-13 was up-regulated by the topical treatment with steroid and OZOILE. Although both treatments induced an increase in IL-13 mRNA levels, OZOILE cream proved to be more effective than mometasone furoate. Indeed, the steroid cream produced a six-fold increase, while the OZOILE caused an 18-fold increase in the transcript levels of IL-13 in comparison to those of untreated tissues (Fig. 2).

We also compared the effects of both creams on the expression of E-cadherin, demonstrating an increase of E-cadherin in tissues from treated patients in comparison to those from untreated ones. In particular, in comparison to foreskin samples from untreated patients, the transcript levels of E-cadherin were increased by fourfold and ninefold by mometasone furoate and OZOILE treatment respectively ($p < 0.001$; Fig. 3).

Discussion

LS of the foreskin is a considered a chronic inflammatory disease of the male genital. The first case was reported in 1962 by Caterall and Oates in a 7-year-old child [21]. Even if the diagnosis can be clinical, most authors agree that it is useful to send the foreskin for histological examination after circumcision [22–24].

The disease can progress to severe complications up to renal failure due to urinary obstruction. For this reason, it is recommended that a prolonged follow-up of patients

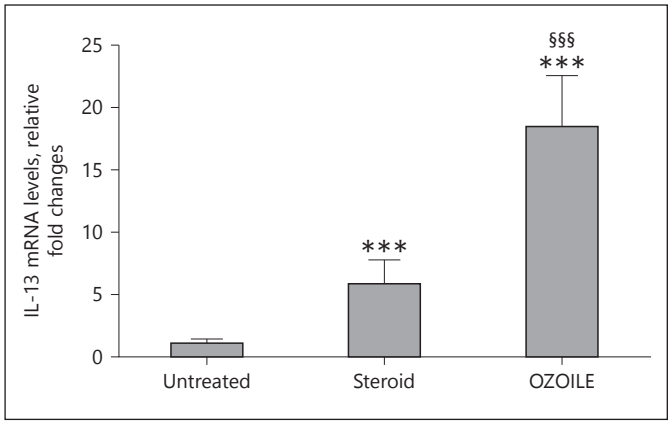

Fig. 2. Changes in mRNA levels of IL-13 in foreskin tissues from patients with LS of the foreskin untreated or treated with topical creams (0.1% mometasone furoate or OZOILE). The results obtained by real-time PCR are expressed as relative fold changes versus foreskin from untreated patients. The data are the means ± standard error of the mean (SEM). *** $p < 0.001$ significant differences in comparison with untreated patients; §§§ $p < 0.001$ significant differences in comparison with foreskins from patients treated with 0.1% mometasone furoate. OZOILE, ozonides with vitamin E acetate.

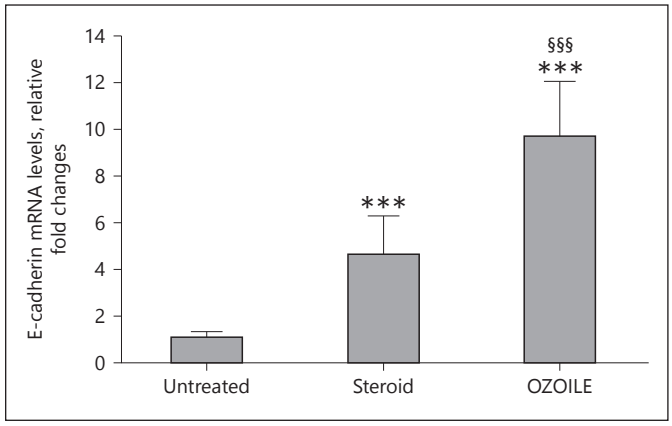

Fig. 3. Analysis of the expression levels of E-cadherin in foreskins from LS affected patients untreated or treated with topical creams (0.1% mometasone furoate or OZOILE). The results obtained by real-time PCR are expressed as relative fold change versus untreated foreskins. The data are the means ± SEM. *** $p < 0.001$ significant differences in comparison with untreated patients; §§§ $p < 0.001$ significant differences in comparison with foreskins from patients treated with 0.1% mometasone furoate. OZOILE, ozonides with vitamin E acetate.

affected by LS of the foreskin be carried out [25]. Therefore, the aim of treatment is abolition of signs and symptoms of disease leading to normal urinary and sexual function and to avoid complications.

Treatment of LS of the foreskin may be surgical with circumcision. This procedure is considered the treatment of choice according to 10 of the 13 articles, including pediatric male patients, reviewed by Celis et al. [8]; in addition, they reported that 5 (50%) of 10 patients who underwent partial circumcision had a recurrence.

Alternative treatment to surgery is topical medical therapy. Several drugs have been proposed. At this time, few large-scale studies or RCTs of LS treatment in children are reported in the literature. Topical corticosteroids have been used before surgery, at the time of surgery, and after surgery [10], and it has been shown that their use can arrest or delay the LS progression [26]. The most commonly used agents include clobetasol propionate and betamethasone valerate [2]. It has been reported that a treatment with clobetasol propionate (0.05%) for 2–3 months is successful in over 90% of cases [27]. A placebo-controlled RCT assessed the efficacy of topical mometasone furoate 0.05 ointment in treating penile LS in 40 boys after 5 weeks' application [26].

Mometasone furoate was found to improve the clinical grade of phimosis in 7 of 17 boys (41%) after 5 weeks treatment. A retrospective study in 21 men with penile LS found clobetasol dipropionate 0.05% cream effective and safe after 7 weeks treatment in about 76% of patients [28]. About 185 males treated with clobetasol propionate 0.05% (for about 12 weeks with decreasing frequency) were analyzed retrospectively, and 60% were successfully treated, with a relapse in some reducing it to 50% success rate; the mean follow-up was 15 months [29].

A 2011 Cochrane by Chi et al. [30] has reviewed 7 randomized clinical trials on the efficacy of common LS treatments in adults without therapeutic indications for pediatric age. In particular, an RCT examining the efficacy of mometasone furoate ointment 0.05% in 40 boys with LS found clinical improvement in 40% [30].

Other drugs have been proposed. Topical testosterone, dihydrotestosterone, and progesterone have been used in the past; in this regard, Chi et al. [30] did not find that topical androgens are effective in the treatment of men and women; therefore, these agents cannot be used in children.

In 2008, Ebert et al. [31] reported their experience in the treatment of penile LS with tacrolimus 0.1% after circumcision, describing 9% of recurrence, which was successfully treated with the same drug. Kirtschig et al.

[16] in their evidence-based guideline on anogenital LS report the use of mometasone and clobetasol in boys only.

Some authors believe that the action of clobetasol propionate therapy in LS seems most likely to be exerted through restoring the balance of collagen synthesis by the fibroblasts and/or reverting the basement membrane distortion by a local induction of immunosuppression and modulation of cytokine expression and surface molecules required for the function of immunocompetent cells [32, 33].

It is known that long-term treatment with corticosteroid can lead to skin atrophy, xerosis, hypopigmentation, burning, irritation, and rarely, suppression of the hypothalamic-pituitary axis [34]. Moreover, Dahlman-Ghozla et al. [28] reported their experience in treatment of penile LS with clobetasol propionate stating that this therapy is safe and effective despite the potential triggering latent infections, most importantly human papillomavirus. In addition, in literature, the phenomenon called "corticosteroid phobia" has been described; this condition is a major reason of anxiety and barrier to effective compliance of parents causing problems to dermatologists and pediatricians to treat children with corticosteroid therapy [35].

In our previous study, we demonstrated that the topical preoperative use of the OZOILE, has a favorable effect on patients undergoing circumcision, inducing a significant reduction of the inflammatory status compared to patients with LS of the foreskin but who had not performed therapy [21].

Ozone effects have been studied for its oxidizing capacity and its disinfectant and sanitizing properties [36]. Ozonides are a class of chemical compounds in which ozone is stabilized by the reaction with unsaturated fatty acids of oils. Ozonides can represent an alternative to pharmacological therapy and they have been used as topical formulation with germicidal properties [36, 37]. In particular, in contact with skin and mucosae, ozonated olive oil, in environments characterized by a protonic increase, such as ischemic, hypoxic, or damaged tissues, releases molecular oxygen driving the production of radical species with the generation of moderate oxidative stress. These effects promote the liberation of growth factors, activation of local antioxidant mechanisms, and tissue repair [36, 37]. Patel et al. [38] have shown the therapeutic effects of ozonated oil on epithelial tissue. In addition, these compounds have been reported to be nontoxic, accelerate wound healing, and exert anti-inflammatory effects [38].

In this study, we compared the effects of OZOILE versus corticosteroid (0.1% mometasone furoate) in the preoperative topical treatment of children affected by LS of the foreskin. Our results demonstrated that the treatment of foreskins affected by LS with OZOILE for 7 days shows similar efficacy of steroid treatment in reducing the inflammatory status, as shown by the diminished expression of TNF-α and IL-1β mRNA levels and the increase in IL-13 transcription in treated foreskins. Particularly, the OZOILE treatment was more effective than the steroid cream in inducing the upregulation of IL-13. This is a relevant result considering the role played by IL-13 in skin health. The IL-13 is an immunoregulatory cytokine produced by several cell types, such as activated Th2 cells, mast cells, and NK cells, and it has been demonstrated to inhibit the production of proinflammatory cytokines and chemokines [39]. Moreover, the IL-13 is produced by the intraepithelial lymphocytes, the specialized tissue-resident T cells, and seems to contribute to the maintenance of healthy epithelium and promote barrier integrity of the epidermis [40]. These effects are associated with the ability of IL-13 to control the differentiation and maturation of keratinocytes necessary to restore skin epithelium.

Based on our previous results demonstrating the property of OZOILE to upregulate the expression of E-cadherin [20], in the present study, we also compared the effects of mometasone furoate and OZOILE on the expression of E-cadherin. E-cadherin is a transmembrane adhesion protein belonging to the cadherin family, involved in cell-to-cell adhesion of the stratified squamous epithelium [41] and is expressed in epithelial tissues [42].

Our results demonstrate a significative increase of E-cadherin in the foreskin of patients treated with both corticosteroid and OZOILE in comparison to tissues from untreated patients. However, the upregulation of E-cadherin induced by OZOILE was greater than that obtained by mometasone furoate treatment.

In our study, we hypothesize that the preoperative topical use of OZOILE and 0.1% mometasone furoate cream has similar and favorable effects in reducing the inflammatory status of foreskins affected by balanitis xerotica obliterans. In addition, OZOILE treatment may be effective in the improvement of skin structure and integrity.

In conclusion, given the data of our study on biochemical changes in tight foreskin, it should be interesting to verify the actual clinical effect of OZOILE as alternative treatment to corticosteroids in children affected by LS of the foreskin. Moreover, encouraged by our preliminary results on molecular levels, we think that a randomized clinical trial, on a larger number of children, verifying the

real effects of preoperative and especially postoperative topical treatment with OZOILE, is necessary to introduce it into therapeutic protocols.

Statement of Ethics

The study protocol has been approved by the Ethical Committee of our hospital and all procedures performed in this study were in accordance with the ethical standards and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent and authorization to use sensitive data were obtained from the parents of all subjects at admission.

Disclosure Statement

The authors declare that they have no conflicts of interest to disclose.

Authors Contribution

C.R. and R.I. conceived the study; D.C. and P.I. designed the experiments; M.C. and N.F. performed the experiments; P.P., E.A., and T.R. collected the samples and clinical data; P.A. and S.A. analyzed the data; M.C. and T.R. wrote the manuscript; D.C. and P.I. provided suggestion and revised the manuscript.

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